



EXPRESS MAIL NO.: EL755732358US
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Andrew J. Flint and Deborah E. Cool
Application No. : 09/788,626
Filed : February 13, 2001
For : IMPROVED ASSAY FOR PROTEIN TYROSINE
PHOSPHATASES

Art Unit : 1741
Docket No. : 200125.401
Date : June 5, 2001

Box Missing Parts
Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT REGARDING SEQUENCE DISCLOSURES

Commissioner for Patents:

In response to the Notice to File Missing Parts dated April 5, 2001, please amend the above-identified application as follows:

In the Specification:

Please insert the enclosed copy of the sequence listing immediately after the "Abstract of the Disclosure" on page 66.

Please replace the paragraph beginning at page 2, line 26, with the following rewritten paragraph:

The PTP family of enzymes contains a common evolutionarily conserved segment of approximately 250 amino acids known as the PTP catalytic domain. Within this conserved domain is a unique signature sequence motif,

[I/V]HCXAGXXR[S/T]G

SEQ ID NO: 36,

that is invariant among all PTPs. The cysteine residue in this motif is invariant in members of the family and is known to be essential for catalysis of the phosphotyrosine dephosphorylation reaction. It functions as a nucleophile to attack the phosphate moiety present on a phosphotyrosine residue of the incoming substrate. If the cysteine residue is altered by site-directed mutagenesis to serine (*e.g.*, in cysteine-to-serine or "CS" mutants) or alanine (*e.g.*, cysteine-to-alanine or "CA" mutants), the resulting PTP is catalytically deficient but retains the ability to complex with, or bind, its substrate, at least *in vitro*.

Please replace the paragraph beginning at page 15, line 13, with the following rewritten paragraph:

As defined herein, a phosphatase is a member of the PTP family if it contains the signature motif [I/V]HCXAGXXR[S/T]G (SEQ ID NO: 36). Dual specificity PTPs, *i.e.*, PTPs which dephosphorylate both phosphorylated tyrosine and phosphorylated serine or threonine, are also suitable for use in the invention. Appropriate PTPs for use in the present invention may be any PTP family member including, but not limited to, PTP1B, PTP-PEST, PTP γ , MKP-1, DEP-1, PTP μ , PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR and PTPH1, and mutated forms thereof.

Please replace the paragraph beginning at page 16, line 4, with the following rewritten paragraph:

As noted above, substrate trapping mutant PTPs are derived from wildtype PTPs that have been mutated such that the wildtype protein tyrosine phosphatase catalytic domain

invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the K_m of the enzyme but which results in a reduction in K_{cat} to less than 1 per minute. Optionally, a catalytic domain cysteine residue is also replaced with a different amino acid, and/or at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In this regard, amino acid sequence analysis of known PTPs reveals the presence of twenty seven invariant residues within the PTP primary structure (Barford et al., 1994 *Science* 263:1397-1404; Jia et al., 1995 *Science* 268:1754-1758), including an aspartate residue in the catalytic domain that is invariant among PTP family members. When the amino acid sequences of multiple PTP family members are aligned (see, for instance, Figure 1A-E in U.S.A.N. 09/334,575; see also, e.g., Barford et al., 1995 *Nature Struct. Biol.* 2:1043), this invariant aspartate residue may be readily identified in the catalytic domain region of each PTP sequence at a corresponding position relative to the PTP signature sequence motif [I/V]HCXAGXXR[S/T]G (SEQ ID NO: 36), which is invariant among all PTPs (see, e.g., WO98/04712; Flint et al., 1997 *Proc. Nat. Acad. Sci.* 94:1680 and references cited therein). However, the exact amino acid sequence position numbers of catalytic domain invariant aspartate residues may be different from one PTP to another, due to sequence shifts that may be imposed to maximize alignment of the various PTP sequences (see, e.g., Barford et al., 1995 *Nature Struct. Biol.* 2:1043 for an alignment of various PTP sequences).

Please replace the paragraph beginning at page 35, line 21, with the following rewritten paragraph:

As a specific example by way of illustration and not limitation, the monoclonal antibody 20G3 (*nanoTools* GmbH, Teningen, Germany) specifically detects the unphosphorylated polypeptide sequence that includes the tyrosine residue at amino acid position 1173, which is a site for autophosphorylation in the ligand-stimulated EGF receptor. Accordingly, fluorescently-labeled phosphopeptides derived from amino acids 1170-1176 of the EGF receptor sequence 1170-1176 (N-A-E-pY-L-R-V, SEQ ID NO: 37) may be substrates for catalytically active PTPs. Furthermore, according to this example, such detectably labeled peptide substrates, upon catalytic dephosphorylation by PTP, would detectably bind to antibody 20G3 and show an increased FP signal commensurate with the degree of dephosphorylation. Termination of dephosphorylation by introduction of a reaction terminator molecule, at an appropriate time that can be determined readily and without undue experimentation based upon the teachings provided herein, then provides a source of fluorescence energy signals for comparison as described herein.

Please replace the paragraph beginning at page 45, line 5 , with the following rewritten paragraph:

For assays of PTP1B many proteins and peptides function as substrates with preferred substrates containing acidic amino acid residues on the N-terminal side of the phosphotyrosine. One of the preferred substrates for PTP1B is derived from autophosphorylation site tyrosine 992 of the EGF receptor. The sequences surrounding this phosphorylation site can be reduced to a hexapeptide without compromising the favorable kinetic properties of this substrate, *i.e.* K_m of ~ 3 μM with a k_{cat} of $72\ s^{-1}$ (Zhang et al., 1994 *Biochemistry* 33:2285-2290). The molecular basis for the recognition of this substrate by PTP1B has been determined by X-ray crystallographic determination of the structure of this peptide (and truncated versions of it) bound to the catalytically inactive C215S mutant of PTP1B (Jia et al., 1995 *Science* 268:1754-1758). The sequence of this peptide, F-erp-P (Figs. 2, 3) D-A-D-E-pY-L-NH₂ (SEQ ID NO: 38) corresponds to residues 988-993 of the human EGF receptor. This peptide is available commercially (Bachem Bioscience Inc., King of Prussia, PA) and can be readily synthesized according to established methodologies.

Please replace the paragraph beginning at page 46 , line 1 , with the following rewritten paragraph:

For comparison to the EGF-receptor derived peptide two other phosphotyrosyl containing substrates were prepared. One, F-IR-P (Figs. 2, 3), which was based on residues 1142-1152 of the human insulin receptor, encompassed three autophosphorylation sites tyrosine 1146, 1151 and 1152. A peptide with only tyrosine 1146 phosphorylated was synthesized at Princeton Biomolecules (Columbus, OH) and labeled with 5'-carboxyfluorescein on the N-terminus, and purified by HPLC. This peptide with sequence T-R-D-I-pY-E-T-D-Y-Y-R (SEQ ID NO: 39) is one of the best reported substrates for PTPs LAR and CD45 with K_m 's of 27 μM and 34 μM respectively (Cho et al., 1992 *Biochemistry* 31:133-138).

Please replace the paragraph beginning at page 46, line 10, with the following rewritten paragraph:

The third peptide utilized in this example, F-lck-P (Figs. 2, 3) corresponded to residues 500-509 of p56lck, the src-like lymphocyte specific protein tyrosine kinase that is a

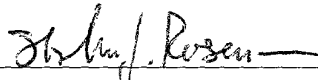
physiological substrate for CD45. The peptide chosen represents the inhibitory phosphorylation site in the C-terminal regulatory segment of p56lck and has a reported K_m of 130 μM toward CD45 (Cho, H., et al., 1992 Biochemistry 31:133-138). Its sequence was A-T-E-G-Q-pY-Q-P-Q-P (SEQ ID NO: 40). This substrate peptide was synthesized, labeled with fluorescein and purified by HPLC by SynPep Corporation, Dublin CA.

REMARKS

The enclosed electronic and paper copies of the Sequence Listing include no new matter that goes beyond the original application as filed, but are supplied to fulfill the requirements as outlined in the Notice to File Missing Parts. Furthermore, the above amendments, which merely direct the insertion of the Sequence Listing and insertion of sequence identifiers, include no matter that goes beyond the original application as filed. Applicant respectfully submits that the above-identified application is now in compliance with 37 C.F.R. §§ 1.821-1.825.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The first of the attached pages is captioned "Version with Markings to Show Changes Made."

Respectfully submitted,
Seed Intellectual Property Law Group PLLC



Stephen J. Rosenman, Ph.D.
Registration No. 43,058

SJR:mls
Enclosures:
Computer Diskette
Declaration Regarding Computer Diskette
Paper Copy of Sequence Listing
701 Fifth Avenue, Suite 6300
Seattle, Washington 98104-7092
Phone: (206) 622-4900
Fax: (206) 682-6031

Application No. : 09/788,626
Docket No. : 200125.401

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Please replace the paragraph beginning at page 2, line 26, with the following rewritten paragraph:

The PTP family of enzymes contains a common evolutionarily conserved segment of approximately 250 amino acids known as the PTP catalytic domain. Within this conserved domain is a unique signature sequence motif,

[I/V]HCXAGXXR[S/T]G

SEQ ID NO: 36,

that is invariant among all PTPs. The cysteine residue in this motif is invariant in members of the family and is known to be essential for catalysis of the phosphotyrosine dephosphorylation reaction. It functions as a nucleophile to attack the phosphate moiety present on a phosphotyrosine residue of the incoming substrate. If the cysteine residue is altered by site-directed mutagenesis to serine (*e.g.*, in cysteine-to-serine or "CS" mutants) or alanine (*e.g.*, cysteine-to-alanine or "CA" mutants), the resulting PTP is catalytically deficient but retains the ability to complex with, or bind, its substrate, at least *in vitro*.

Please replace the paragraph beginning at page 15, line 13, with the following rewritten paragraph:

As defined herein, a phosphatase is a member of the PTP family if it contains the signature motif [I/V]HCXAGXXR[S/T]G (SEQ ID NO: 36). Dual specificity PTPs, *i.e.*, PTPs which dephosphorylate both phosphorylated tyrosine and phosphorylated serine or threonine, are also suitable for use in the invention. Appropriate PTPs for use in the present invention may be any PTP family member including, but not limited to, PTP1B, PTP-PEST, PTP γ , MKP-1, DEP-1, PTP μ , PTPX1, PTPX10, SHP2, PTP-PEZ, PTP-MEG1, LC-PTP, TC-PTP, CD45, LAR and PTPH1, and mutated forms thereof.

Please replace the paragraph beginning at page 16, line 4, with the following rewritten paragraph:

As noted above, substrate trapping mutant PTPs are derived from wildtype PTPs that have been mutated such that the wildtype protein tyrosine phosphatase catalytic domain invariant aspartate residue is replaced with an amino acid which does not cause significant alteration of the K_m of the enzyme but which results in a reduction in K_{cat} to less than 1 per minute. Optionally, a catalytic domain cysteine residue is also replaced with a different amino acid, and/or at least one wildtype tyrosine residue is replaced with an amino acid that is not capable of being phosphorylated. In this regard, amino acid sequence analysis of known PTPs reveals the presence of twenty seven invariant residues within the PTP primary structure (Barford et al., 1994 *Science* 263:1397-1404; Jia et al., 1995 *Science* 268:1754-1758), including an aspartate residue in the catalytic domain that is invariant among PTP family members. When the amino acid sequences of multiple PTP family members are aligned (see, for instance, Figure 1A-E in U.S.A.N. 09/334,575; see also, e.g., Barford et al., 1995 *Nature Struct. Biol.* 2:1043), this invariant aspartate residue may be readily identified in the catalytic domain region of each PTP sequence at a corresponding position relative to the PTP signature sequence motif [I/V]HCXAGXXR[S/T]G (SEQ ID NO:2 36), which is invariant among all PTPs (see, e.g., WO98/04712; Flint et al., 1997 *Proc. Nat. Acad. Sci.* 94:1680 and references cited therein). However, the exact amino acid sequence position numbers of catalytic domain invariant aspartate residues may be different from one PTP to another, due to sequence shifts that may be imposed to maximize alignment of the various PTP sequences (see, e.g., Barford et al., 1995 *Nature Struct. Biol.* 2:1043 for an alignment of various PTP sequences).

Please replace the paragraph beginning at page 35, line 21, with the following rewritten paragraph:

As a specific example by way of illustration and not limitation, the monoclonal antibody 20G3 (*nanoTools GmbH*, Teningen, Germany) specifically detects the unphosphorylated polypeptide sequence that includes the tyrosine residue at amino acid position 1173, which is a site for autophosphorylation in the ligand-stimulated EGF receptor. Accordingly, fluorescently-labeled phosphopeptides derived from amino acids 1170-1176 of the EGF receptor sequence 1170-1176 (N-A-E-pY-L-R-V, ~~SEQ ID NO: 37~~ SEQ ID NO: 37) may be substrates for catalytically active

PTPs. Furthermore, according to this example, such detectably labeled peptide substrates, upon catalytic dephosphorylation by PTP, would detectably bind to antibody 20G3 and show an increased FP signal commensurate with the degree of dephosphorylation. Termination of dephosphorylation by introduction of a reaction terminator molecule, at an appropriate time that can be determined readily and without undue experimentation based upon the teachings provided herein, then provides a source of fluorescence energy signals for comparison as described herein.

Please replace the paragraph beginning at page 45, line 5, with the following rewritten paragraph:

For assays of PTP1B many proteins and peptides function as substrates with preferred substrates containing acidic amino acid residues on the N-terminal side of the phosphotyrosine. One of the preferred substrates for PTP1B is derived from autophosphorylation site tyrosine 992 of the EGF receptor. The sequences surrounding this phosphorylation site can be reduced to a hexapeptide without compromising the favorable kinetic properties of this substrate, *i.e.* K_m of $\sim 3 \mu M$ with a k_{cat} of $72 s^{-1}$ (Zhang et al., 1994 *Biochemistry* 33:2285-2290). The molecular basis for the recognition of this substrate by PTP1B has been determined by X-ray crystallographic determination of the structure of this peptide (and truncated versions of it) bound to the catalytically inactive C215S mutant of PTP1B (Jia et al., 1995 *Science* 268:1754-1758). The sequence of this peptide, F-erp-P (Figs. 2, 3) D-A-D-E-pY-L-NH₂ (SEQ ID NO: 38) corresponds to residues 988-993 of the human EGF receptor. This peptide is available commercially (Bachem Bioscience Inc., King of Prussia, PA) and can be readily synthesized according to established methodologies.

Please replace the paragraph beginning at page 46, line 1, with the following rewritten paragraph:

For comparison to the EGF-receptor derived peptide two other phosphotyrosyl containing substrates were prepared. One, F-IR-P (Figs. 2, 3), which was based on residues 1142-1152 of the human insulin receptor, encompassed three autophosphorylation sites tyrosine 1146, 1151 and 1152. A peptide with only tyrosine 1146 phosphorylated was synthesized at Princeton Biomolecules (Columbus, OH) and labeled with 5'-carboxyfluorescein on the N-terminus, and purified by HPLC. This peptide with sequence T-R-D-I-pY-E-T-D-Y-Y-R (SEQ ID NO: 39)

ID NO: 39) is one of the best reported substrates for PTPs LAR and CD45 with K_m 's of 27 uM and 34 uM respectively (Cho et al., 1992 *Biochemistry* 31:133-138).

Please replace the paragraph beginning at page 46, line 10, with the following rewritten paragraph:

The third peptide utilized in this example, F-lck-P (Figs. 2, 3) corresponded to residues 500-509 of p56lck, the src-like lymphocyte specific protein tyrosine kinase that is a physiological substrate for CD45. The peptide chosen represents the inhibitory phosphorylation site in the C-terminal regulatory segment of p56lck and has a reported K_m of 130 uM toward CD45 (Cho, H., et al., 1992 *Biochemistry* 31:133-138). Its sequence was A-T-E-G-Q-pY-Q-P-Q-P (SEQ ID NO: 40). This substrate peptide was synthesized, labeled with fluorescein and purified by HPLC by SynPep Corporation, Dublin CA.

09798626-060504
T05090" 92988260